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Sixth day of March 2001

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AUSTRALIA
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PROVISIONAL SPECIFICATION

Invention Title: A CELLULAR COMPOSITION INCLUDING NEURAL STEM CELLS (3)

Applicant: MONASH UNIVERSITY

The invention is described in the following statement:

A CELLULAR COMPOSITION INCLUDING NEURAL STEM CELLS (3)

The present invention generally relates to neural stem cells, preferably foetal neural stem cells and their progeny thereof. The present invention provides methods of isolating, culturing and propagating neural stem cells preferably foetal neural stem cells and the development of neural stem cell lines and lineages. The present invention also relates to the use of neural stem cells for gene targeting and gene knockout experiments.

INTRODUCTION

The characterisation and isolation of neural stem cells is useful to understand and treat neurological disorders in mammals. In addition, cell lines based on neural stem cells may be suitable for gene targeting and gene knockout experiments and for nuclear transfer experiments to produce transgenic animals.

Foetal neural stem (FNS) cells are a heterogenous population of glial, astrocyte and neuronal progenitor cells that are capable of differentiating into a variety cell types including neurons. A neural stem cell is an undifferentiated cell that is capable of differentiating into one or more different types of cells. Such stem cells are characterised by having the ability to proliferate, differentiate and are capable of self-renewal. These cells may be derived from various tissues including the brain and/or spinal cord of the embryonic or adult central nervous system.

However, it has been difficult to obtain a neural stem cell line that has the capacity to remain robust and allow for self-renewal and further differentiate *in vitro*.

Several attempts to isolate neural stem cells have been made. US5 928 947 reports methods of isolating and clonal propagation of neural crest stem cells isolated from embryonic tissue. US5 474 930 reports the generation of a non-tumorigenic murine cell line derived from embryonic cells. US6 040 180 reports

the short term propagation (20 days) of rat embryonic stem cells. The source of these specific types of neural stem cells and the methods taught to culture the particular cells are applicable to embryonic tissue. However none of these patents describe or claim, the ability to be able to maintain long-term cultures of neural stems cells.

Therefore, although, culture systems and cell lines have been established from neural stem cells isolated from embryos, it is desirable to develop a neural stem cell line derived from foetal tissue. The neural stem population isolated at this later stage of development has a different phenotype and characteristics to embryonic stem cells. Neural stem cells isolated from foetal tissue are easy to isolate and grow.

The advantage of using neural stem cells is that they are believed to have a greater degree of developmental plasticity and therefore have the ability to generate neural lineages and haematopoietic lineages etc. Therefore, due to the multipotent phenotype of neural stem cells and their ability to readily multiply in a suitable culture they are useful for gene targeting and gene knockout experiments. It would be desirable to develop neural stem cells for gene targeting and gene knockout experiments. Developmental abnormalities associated with nuclear transfer technology using somatic cells have been reported. This results in a high rate of mortality either *in utero* or perinatally. While it is unclear what is causing these defects it is possible that the further a cell has progressed along a differentiation pathway (ie the cells are less plastic) the less able the cell is capable of being reprogrammed. This must occur for nuclear transfer to be successful.

The discussion of documents, acts, materials, devices, articles and the like is included in this description solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia.

Accordingly, it is an object of the present invention to overcome or at least alleviate some of the problems with the prior art.

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a cellular composition comprising one or more cells having a property characteristic of a neural stem cell. Preferably the cells have a property characteristic of a foetal neural stem cell.

In another aspect of the present invention, there is provided a method of preparing a cellular composition comprising a substantially pure population of cells having a property characteristic of a neural stem cell said method comprising:

obtaining a source of neural stem cells;
preparing a suspension of cells from the source;
contacting the suspension of cells with a suitable medium to maintain the neural stem cells in a cell culture;
culturing the cells for a period sufficient to induce the cells to bud from the cell culture;
isolating the budded cells; and
culturing the cells which have budded from the culture.

In another aspect of the present invention, there is provided a media suitable for culturing NSC's, said media including at least one lipid and at least one mitogenic factor in said media.

In another aspect of the present invention, there is provided a genetically modified neural stem cell, said cell comprising a foreign gene which has been introduced into the neural stem cell.

In another aspect of the present invention, there is provided a genetically modified neural stem cell, said cell having a destroyed, modified or deleted

gene. Such genetically modified neural stem cells are useful in gene targeting and gene knockout experiments.

In another aspect of the present invention there is provided a method of preparing a genetically modified animal, said method comprising introducing a neural stem cell into an oocyte or embryo and allowing the resulting embryo to mature to a foetus or animal.

In another aspect of the invention, there is provided a method of treating a neurological disorder, said method comprising introducing a neural stem cell into a host animal to correct the disorder wherein the neural stem cell is capable of replacing neural cells affected by the neurological disorder.

The present invention further includes foetal neural stem cells isolated by the methods hereinbefore described which are transfected with exogenous nucleic acid or are genetically modified by destroying, modifying or deleting genes. Selected foreign nucleic acid may be introduced and/or recombinantly expressed in the cells of the present invention through the use of conventional techniques or the genes may be modified, destroyed or deleted by methods such as point or random mutations.

FIGURES

Figure 1 shows the neural stem cells form a multilayered culture displaying a number of morphologies depending on whether the cells are in direct contact with the tissue culture plate or are part of a secondary layer (Figure 1A). Continued proliferation of the cells results in the formation of budding structures (Figure 1B), which will eventually "hatch" generating balls of cells floating in the media. These balls can be cultured in suspension or disaggregated to for growing on tissue culture plates.

Figure 2 shows that the cells are positive for a number of markers consistent with neural stem cells including nestin (Figure 2A) and vimentin (Figure 2B).

DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a cellular composition comprising one or more cells having a property characteristic of a neural stem cell. Preferably the cells have a property characteristic of a foetal neural stem cell.

The neural stem cells of the present invention may be characterised by their ability to grow indefinitely in tissue culture without undergoing transformation and retain some degree of developmental plasticity. The phenotype of the neural stem cells do not change over long term culturing and the plasticity of the neural stem cells make them suitable for nuclear transfer experiments and various other applications such as gene knockout experiments.

Like all neural stem cells, or preferably foetal neural stem cells, these cells have the capacity to differentiate into one or more different types of cells when placed in differentiating conditions. The types of cells, which may result from differentiation, include haematopoietic stem cells and their lineages and neural stem cells and their lineages.

The neural stem cells, and preferably the foetal neural stem cells have the capacity to grow indefinitely in tissue culture and this means that they can remain undifferentiated. The degree of plasticity means that these cells have the ability to generate multiple cell types and the cells of the present invention may be identified by these characteristics.

The neural stem cells, and preferably the foetal neural stem cells also have the characteristic that they are trypsin-sensitive as even mild trypsin destroys the cells. They are also epidermal growth factor (EGF) dependant for cell division. Removal of EGF from the medium stops cell division in the cells and induces quiescence of the cells. The quiescence of the cells may be reversed by the addition of EGF.

Another important feature of the present cells is their capacity to culture indefinitely and "bud off" into the media. This feature can be utilised as a method of propagation of the cells. Each bud comprises a plurality of cells which may be cultured to provide an isolated and purified population of the neural stem cells. Preferably they are foetal neural stem cells.

The cells may also be identified by cell markers. Apart from the standard neural cell markers, other markers including but not limited to nestin, vimentin etc, may be used to identify the neural stem cells, preferably foetal neural stem cells. Accordingly they are preferably foetal neural stem cell markers.

In another aspect of the present invention, there is provided a method of preparing a cellular composition comprising one or more cells having a property characteristic of a neural stem cell said method comprising:

obtaining a source of neural stem cells;

preparing a suspension of cells from the source;

contacting the suspension of cells with a suitable medium to maintain the neural stem cells in a cell culture;

identifying the cells which have the properties of neural stem cells; and

isolating the cell with properties of neural stem cells.

Preferably the neural stem cell is a foetal neural stem cell having the properties as described above.

The source of neural stem cells may derive from any animal that has a nervous system. Preferably the animal is a mammal including but not limited to murine, bovine, ovine, porcine, equine, feline, endangered species, live stock or may derive from marsupials including kangaroos, wombats.

Neural stem cells may be collected from any embryonic stage of development after that the neural stem cells are present. More preferably the source of neural stem cells is from a foetus which is differentiated at a stage after the embryonic stage. The whole foetus or a part thereof containing neural cells may be used as a source of the neural cells. Preferably the head or spinal cord

of the foetus provide the source of neural stem cells. More preferably, the head is used as a source of foetal neural stem cells.

Preferably the cells are obtained from rat foetuses and more preferably from the head of a rat foetus. It has been found that foetus obtained from Sprague-Dawley rats provides a reliable source of foetal neural stem cells.

The suspension may be prepared by obtaining the tissue, preferably from the head or spinal cord of the foetus which may be homogenized preferably in the absence of trypsin or with minimal trypsin, preferably 0.1%. The time of treatment with trypsin should not exceed 15 minutes at 37°C. After treatment with trypsin, it is preferred to further use a trypsin inhibitor, such as soyabean trypsin inhibitor preferably constituted at approximately 1mg/ml in medium such as DMEM/F12 added 1:1 (v/v) with the trypsin solution. However, other media as discussed below may be used.

In another aspect of the present invention, there is provided a media suitable for culturing NSCs, said media including at least one lipid and at least one mitogenic factor within said media.

A suitable medium to maintain the cells in culture is a medium which can perpetuate the cultured NSCs as herein described, most preferably they are cultured indefinitely.

The media may contain known components that in combination, support the growth of the cultured neural stem cells or preferably the foetal stem cells. The media may include other nutrients, buffers, hormones, salts, antibiotics, proteins, growth factors and enzymes. A suitable medium may contain DMEM/F12 with Hepes, glucose, bicarbonate, antibiotics (penicillin/streptomycin and ampicillin). Additional supplements may be added including insulin, transferrin, epidermal growth factor (EGF), lipids (cholesterol, triglyceride, phospholipids) and fibronectin.

A medium which contains at least a combination of one or more mitogenic factors and lipids is found to be most preferred for culturing the NSCs, more particularly for culturing the NSCs indefinitely. Suitable mitogenic factors may be selected from the group including, but not limited to, bFGF, EGF and PDGF. These factors may be used alone or in combination with the lipids providing both lipids and mitogenic factors are included in the media. EGF and/orbFGF are mostly preferred as mitogenic factors in the media.

Some components may be substituted for others (eg insulin-like growth factors for insulin; transforming growth factor alpha for epidermal growth factor; bovine serum albumin containing lipids; polylysine for fibronectin; and iron salts for transferrin). Further, other factors might be added to the culture medium, such as tumor promoters, additional hormones and/or growth factors, bovine serum albumin, low concentrations of serum or plasma, or modified plasma preparations with reduced inhibitory activity. Fibronectin might be eliminated from the culture medium formulation to obtain anchorage-independent growth of the present cell lines. Alteration of culture medium components may also allow derivation of sublines of the non-tumorigenic cell lines of the present invention or their equivalent. In addition, other supplements may be added to the medium formulation to enhance protein production from a particular foreign gene construct (for example, addition of steroid hormones where the foreign gene is operably linked to a steroid hormone-responsive promoter).

More preferably, the media contains at least a cell survival factor, such as transferrin, insulin, growth factors such as EGF, bFGF (FGF-2) or PDGF, lipids and selenium.

For example, a suitable media for growing and maintaining neural stem cells may comprise Neurobasal-A media® (Life Technologies), containing Insulin-Transferrin-Selenium (Life Technologies) – 1:100; EGF 2-20–ng/ml; bFGF 2-10 ug/ml, Chemically defined lipid concentrate (Life Technologies) – 1:100; N-2 supplement (Life Technologies) 1:100; B-27 supplement (Life technologies) 1:100, L-glutamine 1-2 mM.

Methods of identifying the cells which have the characteristics of neural stem cells may be any method known to the skilled addressee for detecting the properties listed above. For instance for detecting cell markers, antibodies (monoclonal or polyclonal) are available to identify them. Testing for EGF and trypsin sensitivity may be simply tested in the presence or absence of these factors.

Methods of isolation may be employed based on the methods of identification. For instance, antibodies may be used to select those neural stem cells having the appropriate markers, alternatively suitable cell culture conditions may be used to obtain cells with the morphology of the neural stem cells of the present invention.

In another aspect of the present invention there is provided a cellular composition comprising a substantially pure population of cells having a property characteristic of a neural stem cell. Preferably the cells have a property characteristic of a foetal neural stem cell.

In another aspect of the present invention, there is provided a method of preparing a cellular composition comprising a substantially pure population of cells having a property characteristic of a neural stem cell said method comprising:

obtaining a source of neural stem cells;

preparing a suspension of cells from the source;

contacting the suspension of cells with a suitable medium to maintain the neural stem cells in a cell culture;

culturing the cells for a period sufficient to induce the cells to bud from the cell culture;

isolating the budded cells; and

culturing the cells which have budded from the culture.

The neural stem cells of the present invention have the characteristic of being able to "bud off" into the media. These can be seen with the naked eye. The

buds may be collected and spun down. The buds may be disaggregated by any method available to the skilled addressee. However, vigorous pipeting can disaggregate the buds to provide separate cells. Prolonged use of trypsin is discouraged as the cells are sensitive to trypsin. Once disaggregated, the cells may be inoculated into a fresh medium, preferably in a media described above. Therefore the present invention also relates to the long-term clonal expansion or propagation of neural stem cells, preferably foetal neural stem cells.

The cells may be passaged using trypsin for a short period. Cells are first washed with PBS to remove media. The cells may be loosened from the plate using a trypsin solution for a minimal period at 37°C, usually less than 2 min. Preferably the cells be free of the tissue culture plate. However, they do not need to be totally disaggregated. The trypsin may be neutralised using soyabean trypsin inhibitor, preferably at: 1 mg/ml made up in the media being used to culture cells added 1:1 (v/v) to the trypsin solution. The cells may be spun down at low speed in a centrifuge, the media removed and the cells resuspended in fresh media and plated in new fibronectin-treated tissue culture plates. The cells may be split 1:4. Preferably the cells are maintained at a minimum plating density of 1×10^4 cells/cm^2.

The cells may be frozen preferably in 90% FCS, 10% DMSO or by any methods available to the skilled addressee which would be suitable for freezing cells.

The neural stem cells of the present invention have the capacity to grow indefinitely without undergoing transformation and retain a degree of plasticity. This can be achieved by culturing and propagating the cells as described above.

Accordingly, the present invention also provides an isolated neural stem cell prepared by the method described above. Preferably it is a foetal neural stem cell.

In another aspect of the present invention, there is provided a genetically modified neural stem cell, said cell comprising a foreign gene which has been introduced into the neural stem cell.

In another aspect of the present invention, there is provided a genetically modified neural stem cell, said cell having a destroyed, modified or deleted gene. Such genetically modified neural stem cells are useful in gene targeting and gene knockout experiments.

A genetically modified neural stem cell refers to a cell into which a foreign (ie non-naturally occurring) nucleic acid, eg, DNA, has been introduced. The foreign nucleic acid may be introduced by a variety of techniques, including, but not limited to, calcium-phosphate-mediated transfection DEAE-mediated transfection, microinjection, retroviral transformation, electroporation, immunoporation, protoplast fusion and lipofection. The genetically modified cell may express the foreign nucleic acid in either a transient or long-term manner. In general, transient expression occurs when foreign DNA does not stably integrate into the chromosomal DNA of the transfected cell. In contrast, long-term expression of foreign DNA occurs when the foreign DNA has been stably integrated into the chromosomal DNA of the transfected cell.

Foreign (heterologous) nucleic acid may be introduced or transfected into neural stem cells. A multipotent neural stem cell which harbours foreign DNA is said to be a genetically modified cell. The foreign DNA may be introduced using a variety of techniques. In a preferred embodiment, foreign DNA is introduced into multipotent neural stem cells using the technique of retroviral transfection. Recombinant retroviruses harbouring the gene(s) of interest are used to introduce into multipotent neural stem cells using the technique of retroviral transfection. Recombinant retroviruses harbouring the gene(s) of interest are used to introduce marker genes, such as but not limited to βgalactosidase (lacZ) gene, or oncogens. The recombinant retroviruses are produced in packaging cell lines to produce culture supernatants having a high titre of virus particles (generally 10.sup.5 to 10.sup.6 pfu/ml). The recombinant viral particles are used to infect cultures of

the neural stem cells or their progeny by incubating the cell cultures with medium containing the viral particles and 8.μ.g/ml polybrene for three hours. Following retroviral infection, the cells may be rinsed and cultured in standard medium. The infected cells may be then analysed for the uptake and expression of the foreign DNA. The cells may be subjected to selective conditions which select for cells that have taken up and expressed a selectable marker gene.

The present invention accordingly includes foetal neural stem cells isolated by the methods hereinbefore described which are transfected with exogenous nucleic acid. Selected foreign nucleic acid may be introduced and/or recombinantly expressed in the cells of the present invention through the use of conventional techniques.

In another aspect of the present invention there is provided a method of preparing a genetically modified animal, said method comprising introducing a neural stem cell into an oocyte or embryo and allowing the resulting embryo to mature to a foetus or animal.

The neural stem cell is preferably a foetal neural stem cell prepared by the methods described above. In a preferred aspect the neural stem cell is a genetically modified neural stem cell as described above having a gene inserted, deleted or destroyed. The foreign gene may be a gene encoding a desired product preferably to induce a desired characteristic in the genetically modified animal or to generate a gene knockout model wherein the gene is absent.

Accordingly, the present invention preferably provides knockout animals which are useful for research in gene function, diseases, drug therapies and gene development of animal strains having knockout genes.

In another aspect of the invention, there is provided a method of treating a neurological disorder, said method comprising introducing a neural stem cell

into a host animal to correct the disorder wherein the neural stem cell is capable of replacing neural cells affected by the neurological disorder.

The neural stem cell is preferably a foetal neural stem cell as described above. For treating a neurological disorder where neural cells are destroyed, the neural cells may be capable of regenerating the neural tissue. Alternatively, if a foreign gene encoding a protein beneficial for treating the neurological disorder is inserted into a neural stem cell or preferably a foetal neural stem cell, then the genetically modified neural stem cell may be introduced into the patient in need of regeneration and treatment of the neurological disorder. Preferably, the neurological disorder is Parkinson's disease.

The present invention also includes the use of foetal neural stem cells in a wide range of applications including but not limited to transplantation, nuclear transfer and gene targeting and gene knockout experiments, the generation of transgenic animals and the construction of animal models.

Throughout the description and claims of the specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1 - Preparation of foetal neural stem cells

Tissue culture plates were pre-coated with fibronectin at 20μg/ml in DMEM/F12 for 1-2 hours at 37°C 5% CO_2. (Enough volume was used to cover the surface). The fibronectin was aspirated and washed and then plated in DMEM/F12. This preparation can be stored at room temp for several days.

A pregnant rat (eg. Sprague-Dawley) was humanely killed at 10.5-16.5 days gestation by CO_2 asphyxiation. Foetuses were removed and placed into a tube with PBS containing penicillin/streptomycin.

Membranes from the foetuses were removed and their heads were separated from their bodies. The pooled foetal heads were placed into a small dish (6cm) and the tissue was minced with a blunt object (the tip of a syringe) until it was homogeneous in size. A syringe was used to aspirate the minced tissue which was then transferred into a tube. The dish was washed with 5-10 ml PBS and then aspirated into the syringe and pooled into the tube containing the tissue.

The minced tissue was left to settle at the bottom of the tube for a few minutes and was carefully aspirated off the liquid. The tissue was washed with fresh PBS until it was reasonably clear (approximately 2 washes). 5 ml of Trypsin 0.1% in versene), was added to the tissue and the tube was placed into a 37°C water bath, at 37°C for no longer than 15 min (The tubes were mixed occasionally). The tissue was allowed to settle down to the bottom of the tube and the cell suspension was transferred into a centrifuge tube. The tissue was washed in 5ml soyabean trypsin inhibitor (1mg/ml in DMEM/F12), and the cell suspension was pooled with the trypsin cell suspension.
The cells were spun down and resuspend in a small volume of media and live cell numbers were estimated using a haemocytomer and staining of the cells with trypan blue.

The cells were placed onto fibronection pre-coated plates at a density of approximately 1.5 x 10^5 cells/cm^2.

Example 2 - Defined Medium for culturing foetal neural stem cells

The FNS cell medium suitable for the present invention comprises Dulbecco-modified Eagle's medium (DMEM) comprising 15 mM 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid, 4.5g/l glucose, 1.2g/l Bicarbonate, 200 U/ml Penicillin, 200 µg/ml Streptomycin, 25µg/ml Ampicillin; and the following additional components are added prior to use of the media:

Bovine insulin (10µg/ml), Human transferrin (25µg/ml), Mouse EGF (2-20 ng/ml), Sodium selenite 10 nM, and Human HDL (freshly isolated) 25µg/ml. The EGF growth factor may be substituted with bFGF (FGF-2) or any other suitable mitogenic growth factors.

Example 3 – Alternate defined medium for culturing of foetal neural stem cells

Neurobasal-A media® (Life Technologies), containing Insulin-Transferrin-Selenium (Life Technologies) – 1:100; EGF 2-20 ng/ml; Chemically defined lipid concentrate (Life Technologies) – 1:100; N-2 supplement (Life Technologies) 1:100; B-27 supplement (Life technologies) 1:100, L-glutamine 1-2 mM.

Example 4 - Culturing and Passaging of foetal, neural stem cells

When the cells were cultured only half of the media was changed at once. The media was taken off and the cells were spun down, half of the fresh media and half of the conditioned media was added onto cells. A half media change was carried out if the cells were sub-confluent and had not formed loose neuronal buds. Once the cells reached ~80% confluence a complete change of media was performed.

When the cells were passed over several days they were allowed to plate down and form neuronal cells that "bud off" into the media. (These can be seen with the naked eye). The media (containing the buds) were pipetted of and spun down. The media was retained for diluting with fresh media. The cell buds were disaggregated by pipetting vigorously with a yellow tip (with care not to cause bubbles). A fresh flask was inoculated with these disaggregated buds at an approximately a 1 in 3 dilution.

The cells were frozen down in, DMEM/F12 media containing 10% DMSO.

Although the specific procedure and methods described herein are exemplified using rat neural stem cells, they are merely illustrative for the practice of the invention. Analogous procedures and techniques are equally applicable to all mammalian species, including but not limited to human, non-human primate, feline, canine, bovine, porcine, endangered species, live stock etc.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 31 October 2000
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
MONASH UNIVERSITY David B Fitzpatrick

Figure 1A

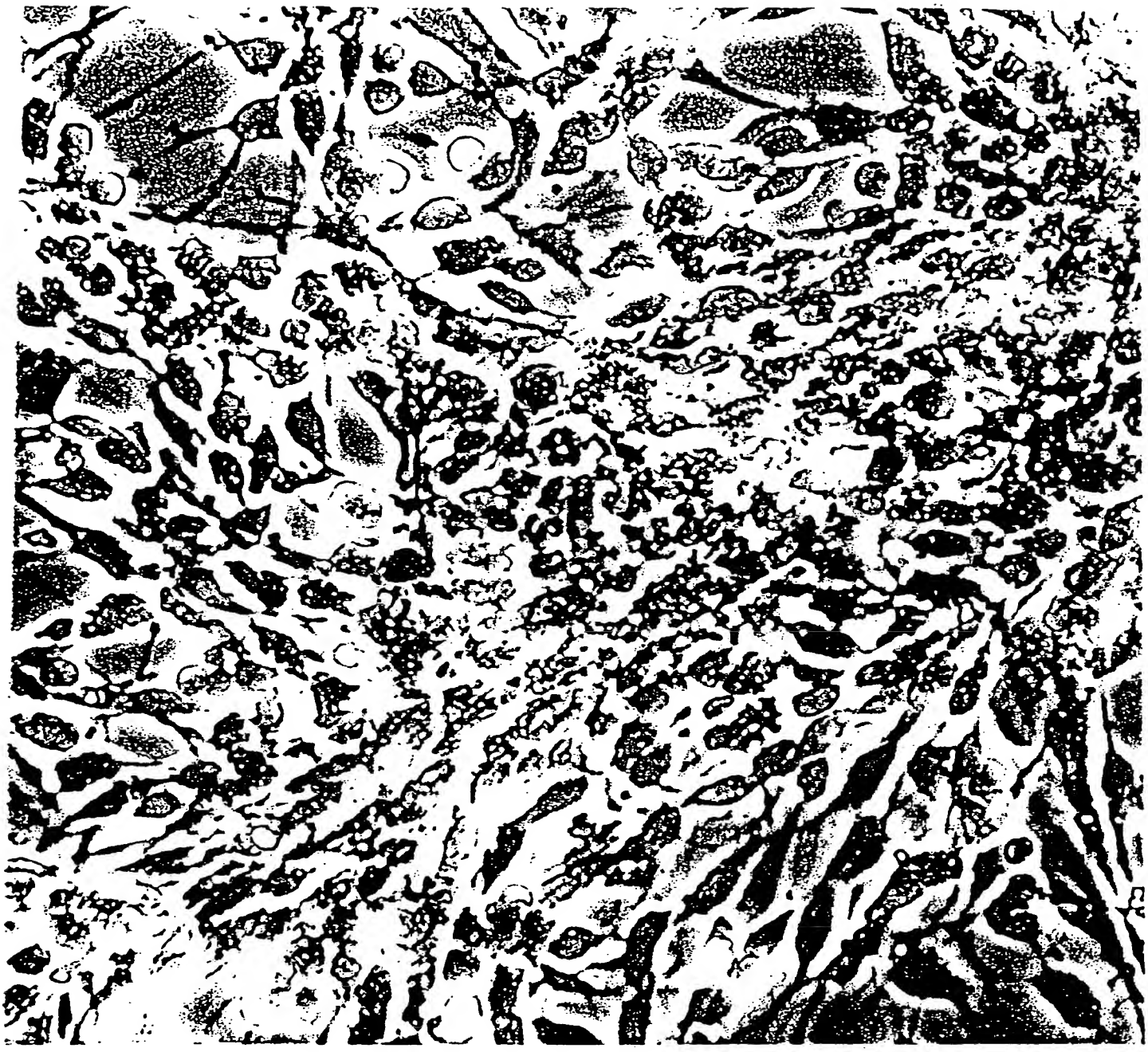

Figure 1B

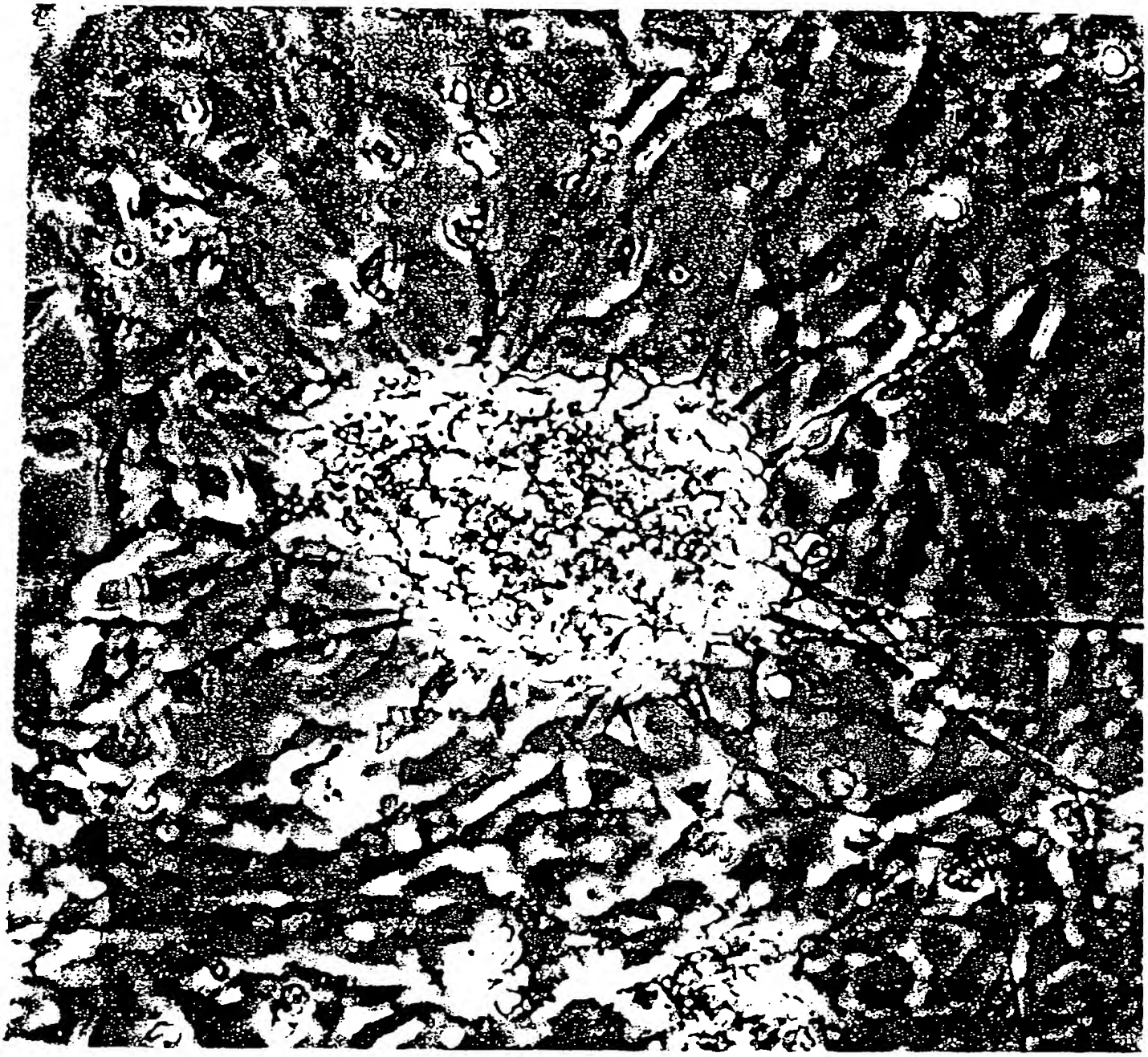

Figure 2A

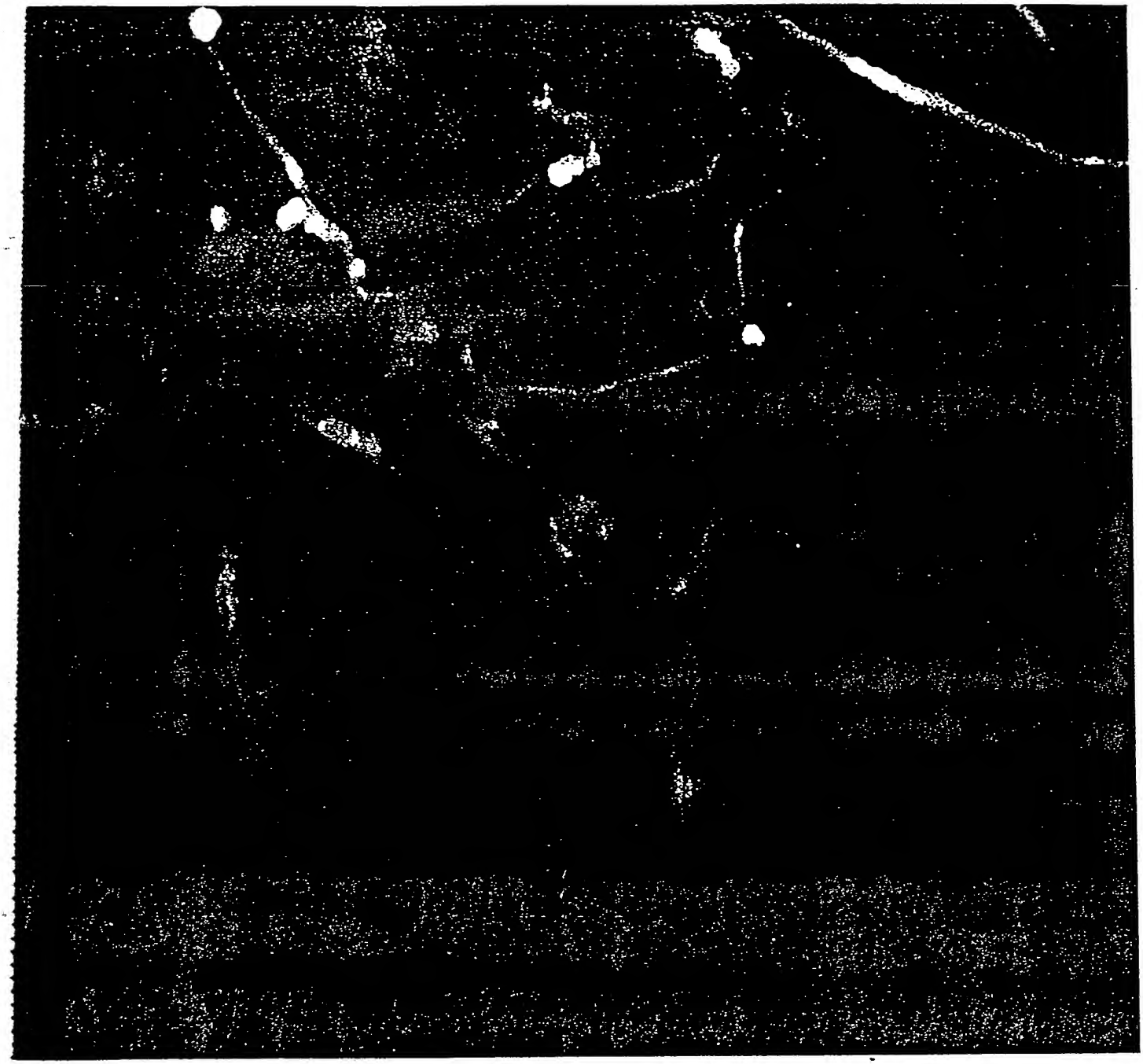

Figure 2B